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Aggregation of Lens Crystallins in an In Vivo Hyperbaric Oxygen Guinea Pig Model of Nuclear Cataract: Dynamic Light-Scattering and HPLC Analysis

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Abstract

Purpose—The role of oxygen in the formation of lens high-molecular-weight (HMW) protein aggregates during the development of human nuclear cataract is not well understood. The purpose of this study was to investigate lens crystallin aggregate formation in hyperbaric oxygen (HBO)–treated guinea pigs by using in vivo and in vitro methods.

Methods—Guinea pigs were treated three times weekly for 7 months with HBO, and lens crystallin aggregation was investigated in vivo with the use of dynamic light-scattering (DLS) and in vitro by HPLC analysis of water-insoluble (WI) proteins. DLS measurements were made every 0.1 mm across the 4.5- to 5.0-mm optical axis of the guinea pig lens.

Results—The average apparent diameter of proteins in the nucleus (the central region) of lenses of HBO-treated animals was nearly twice that of the control animals ($P < 0.001$). Size distribution analysis conducted at one selected point in the nucleus and cortex (the outer periphery of the lens) after dividing the proteins into small-diameter and large-diameter groups, showed in the O_2-treated nucleus a threefold increase in intensity ($P < 0.001$) and a doubling in apparent size ($P = 0.03$) of large-diameter aggregate proteins, compared with the same control group. No significant changes in apparent protein diameter were detected in the O_2-treated cortex, compared with the control. The average diameter of protein aggregates at the single selected location in the O_2-treated nucleus was estimated to be 150 nm, a size capable of scattering light and similar to the size of aggregates found in human nuclear cataracts. HPLC analysis indicated that one half of the experimental nuclear WI protein fraction (that had been dissolved in guanidine) consisted of disulfide cross-linked 150- to 1000-kDa aggregates, not present in the control. HPLC-isolated aggregates contained αA-, β-, γ-, and ζ-crystallins, but not αB-crystallin, which is devoid of −SH groups and thus does not participate in disulfide cross-linking. All ζ-crystallin present in the nuclear WI fraction appeared to be there as a result of disulfide cross-linking.

Conclusions—The results indicate that molecular oxygen in vivo can induce the cross-linking of guinea pig lens nuclear crystallins into large disulfide-bonded aggregates capable of scattering light. A similar process may be involved in the formation of human nuclear cataract.

Cataract affects more than 20 million adults 40 years of age and older in the United States[1] and is the leading cause ofhuman blindness worldwide.[2] Nuclear cataract, an opacity that forms in the center of the aging ocular lens, is a major type of this disorder[3,4] and is the type of cataract most likely to necessitate surgery.[5] It has also been reported to be a significant

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predictor of mortality.[6] Although the specific mechanism of formation of nuclear cataract is unclear, it is known to be linked with substantial oxidation of the major proteins of the lens, the crystallins. Crystallins remain in the center of the lens for many years because there is no protein renewal in this region. Oxidation of cysteine residues is a particularly significant feature of nuclear opacity.[7–9] More than 90% of the nearly 50-mM level of normally reduced −SH groups in the human lens become oxidized in mature nuclear cataracts,[10,11] producing disulfide cross-linking of crystallins, aggregate formation, and protein insolubilization.[12] To remain transparent, the center of the lens must continuously maintain −SH groups in a nearly 100% reduced state.[13–15] Why the mammalian lens, with its high concentration of the longest-lived proteins in the body, would require such a substantial level of potentially oxidizable cysteine residues is an important, unanswered question in lens and cataract research.[16]

The role that molecular oxygen may play in the formation of nuclear cataract is of current interest. The partial pressure of O_2 in the center of the lens is normally low, approximately 10 mm Hg or 1%,[17] due in part to metal-catalyzed reactions of the molecule with ascorbate and glutathione (GSH).[13,18] Much of the O_2 that enters the anterior side of the lens from the aqueous humor is consumed by mitochondria located in the lens epithelium and superficial cortex.[19] With age, the level of GSH in the lens nucleus falls steadily,[20,21] making this region of the organ more susceptible to O_2-induced damage. Age-related liquefaction of human vitreous humor may permit increased migration of O_2 from the retinal circulation through the vitreous space into the posterior side of the lens, possibly accelerating the formation of nuclear cataract.[22,23] This type of mechanism may also be associated with the lens nuclear opacity that frequently follows the surgical procedure of vitrectomy in patients.[17,23] Additional evidence to link O_2 with the formation of nuclear cataract comes from the therapeutic treatment of patients with hyperbaric oxygen (HBO), exposures that when conducted over extended periods of time can cause lens nuclear opacity.[24] Experimental treatment of guinea pigs with HBO has been shown to induce an increased level of light-scattering in the center of the lens,[25] a known precursor to nuclear cataract.[26] HBO treatment also accelerates various age-related effects in the guinea pig lens nucleus, including the insolubilization of cytoplasmic protein, formation of protein disulfide, oxidation of membrane lipids and degradation of cytoskeletal proteins and the membrane protein aquaporin-0 (formerly known as MIP26).[25,27,28]

Electron microscopic analysis of human nuclear cataracts, obtained after surgical extraction, has revealed no extensive cellular damage occurring in the lens center,[29,30] suggesting that aggregation of crystallins, rather than damage to the nuclear fiber cell membrane, is the major contributor to increased light-scattering in this type of opacity. Theoretical considerations have predicted that aggregates with diameters of 20 to 200 nm or greater and molecular masses >50 $\times 10^6$ Da would be needed to produce significant light-scattering in the lens.[31–33] (The largest oligomer in the normal, clear lens is α-crystallin, with a size of 10 to 15 nm and an average molecular mass of 8×10^5 Da.[34–36]) Fourier analysis of electron micrographs have indicated the presence of aggregates >100 nm in diameter in the central region of both human nuclear cataracts and lenses of guinea pigs treated extensively with HBO.[37,38] Dynamic light-scattering (DLS) has been used for nearly 30 years to investigate the aggregation of lens proteins, both in vitro in intact lenses[39–42] and in vivo.[43–48] DLS[49] employs a weak incident laser beam to measure the Brownian movement of lens proteins. The technique is emerging as a valuable diagnostic tool for early detection of ocular diseases such as cataract.[50,51] In vivo DLS analyses conducted on human subjects have shown increased aggregation of lens proteins with age,[47] and detected 100-nm aggregates in the lens nucleus of patients with early cataracts.[46] Experimentally, in vivo DLS has been used to demonstrate an increase in protein size in the lenses of x-rayed rabbits, well before the formation of mature cataract.[44,48] In the present study, we combined in vivo DLS with HPLC analysis to investigate the formation of crystallin aggregates in lenses of guinea pigs treated with HBO. The HPLC work focused on the analysis of water-insoluble (WI) proteins of the guinea pig lens. In a recent HPLC/mass spectrometry

study, it was found that disulfide is a major modifier of proteins present in the WI fraction of old, normal human lenses.[52]

Materials and Methods

Animals

Male retired breeder Hartley guinea pigs, initially 17 to 18 months old, were obtained from Kuiper Rabbit Ranch (Indianapolis, IN). The animals were held for 1 to 2 weeks before HBO treatment, to allow recovery from the stress of shipment and to identify the healthiest animals for the study. During this time, the lenses of the guinea pigs were examined carefully by slit lamp biomicroscopy, and animals with cortical or nuclear opacities were excluded. Guinea pigs treated with HBO have been shown to grow at a normal rate.[25] All animal care and work performed in this study conformed to U.S. Department of Agriculture standards and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

HBO Treatment

Guinea pigs were treated in a pressure vessel 114 cm long and 46 cm in diameter (Amron, Escondido, CA). The vessel had a fully opening hinged door at one end and, at the opposite end, a 15-cm viewport for observing the animals during the experiments. Light from a 50-W tungsten halogen projection lamp, located outside the chamber, was led inside through an acrylic light pipe and was kept on during each treatment. Fourteen animals were treated at a time—seven in each of two Lucite boxes with screened tops. Plastic trays containing water were placed inside the chamber to add humidity. Soda lime (Sodasorb; WR Grace, Lexington, MA) was added to absorb CO_2, and ice was added to maintain the temperature below 23°C. After the chamber was sealed, it was flushed for 5 to 10 minutes with approximately 1 volume of 100% O_2 (USP Grade Medical Gas; Praxair, Danbury, CT) which was vented outside the building. The pressure was then raised during 15 minutes to 2.5 atm absolute (ATA; 22.3 psig [pounds per square inch gauge] or 51 ft of sea water) of O_2. After 1.25 hours, the chamber was flushed with approximately 1 volume of fresh 100% O_2, while the pressure was maintained at 2.5 atm. At the end of a 2.5-hour holding period, the pressure was released over a 15-minute period to 1 ATA (0 psig), and the animals were removed. The guinea pigs were treated three times per week, on alternate days, at approximately the same time each day, for a total of 84 treatments over a 7-month period. Age-matched control animals were included with each group of O_2-treated animals. The transparency of lenses of control and HBO-treated guinea pigs was assessed with a slit lamp microscope (Carl Zeiss Meditec, Thornwood, NY) after induction of full mydriasis with tropicamide (1%) and phenylephrine (10%). The results were documented by photography. After animals were killed by CO_2 asphyxiation, the eyes were enucleated, and the lenses removed in a posterior approach.

DLS Analysis

Static light-scattering (SLS) and DLS measurements were conducted on eyes of control and experimental guinea pigs in vivo by using a compact fiber optic laser probe. The experimental setup, as well as the probe design and its application for three-dimensional scanning of the eyes of animals have been published.[48,53,54] Before the measurements, the eyes of the guinea pigs were dilated with a 1% solution of tropic-amide (Mydriacyl; Alcon Pharmaceutical Co., Fort Worth, TX), and the animals were anesthetized with intramuscular injections of xylazine (8 mg/kg) and ketamine (40 mg/kg), administered 10 minutes apart. A laser power of 100 μW with a wavelength of 640 nm was used for the measurements, which were conducted in a dark room. Each animal was positioned so that the laser beam passed through the center of the eye along the optical axis of the lens. The scattered light was collected at 154°. The corneal surface of each eye under analysis was kept moist by topical application of 0.9% saline.

For SLS measurements, the cornea of the eye was detected by moving the laser light probe toward the eye until a sharp peak was observed in collected light intensity (measured in *K* counts/s or 1000 photon counts per second) as a result of reflection of the light by the cornea. At a threshold of ≥10,000 *K* counts/s, the movement of the motorized actuator was automatically stopped, and the surface of the cornea was used as the point of origin for the SLS scan. The laser light probe was moved at a velocity of 0.01 mm/s from the cornea to the posterior capsule of the lens, a distance of approximately 6 mm. A complete SLS scan of the guinea pig eye took approximately 13 minutes.

DLS measurements, conducted to determine the relative sizes of proteins in experimental lenses compared with controls, were performed immediately at the end of each SLS scan, without changing the position of the laser light beam within the eye. The anterior capsule of the lens, which gave a high intensity of counts relative to the aqueous humor, was used as the zero position for each DLS analysis. Measurements were made every 0.1 mm (5 seconds each) across the 4.5- to 5.0-mm optical axis of the guinea pig lens, for a total of ~50 measurements per lens. The analysis volume of focused laser light at each measurement point was 8×10^{-6} mm^3 ($20 \times 20 \times 20$ μm). The instrument performance was checked by measuring the DLS of standard suspensions of polystyrene beads having an 80-nm diameter. Each set of DLS measurements took approximately 10 minutes per lens to complete. At each of the 50 locations in the lens, the instrument made 10 to 15 measurements of protein diameter in a 5-second period and provided an average value. In this study, DLS data are not expressed as absolute sizes of the lens proteins, but instead as arbitrary units of diameter. In this way, relative sizes of the lens proteins can be compared in different locations in the lens, between control and HBO-treated. The DLS technique can be used to measure absolute protein size only when the proteins are noninteracting and in dilute solution. Because of the very high concentration of crystallins present in the intact lens (>300 mg/mL), many Coulombic and Van der Waals interactions are produced, which significantly slow the diffusion of proteins, increasing their apparent size. Bettelheim[55] has discussed the spuriously large measurements of proteins that can result from attempting to measure absolute protein size in intact lenses with DLS. Some earlier DLS studies have assumed that the lens protein–water system behaves as a dilute solution,[40,41] but such an assumption was not made in the present work.

HPLC Analysis

For HPLC analysis, control and experimental guinea pig lenses were frozen rapidly in crushed dry ice and separated into equatorial cortex (the periphery of the lens) and nucleus (the center of the lens) with the use of a cork borer to produce a cylinder and a doughnut-shaped segment. The cylinder was trimmed by 10% of the total length on each end (the trimmed portions were discarded) to produce the nucleus, amounting to 25% of the total lens weight. The equatorial cortex (the doughnut) amounted to 55% of the total weight. The tissues were homogenized (100 mg wet weight of lens per milliliter buffer) at 0 ° to 4°C in 5 mM Tris/HCl buffer (pH 7.5) containing 50 mM NaCl and 2 mM EDTA. Care was taken not to produce any artifactual disulfide by inclusion of EDTA in the homogenizing buffer and conducting the homogenization in an N_2 atmosphere. In the initial experiments, no agents were added to the mixture to reduce endogenous disulfide. After centrifugation of the homogenate for 25 minutes at 14,000 rpm, the WI pellets were isolated and washed twice with cold 50 mM 2-(*N*-morpholino)ethane sulfonic acid (MES) buffer (pH 6.2), containing 1 mM EDTA. The pellets were dissolved in 50 mM MES buffer (pH 6.2), containing 6 M guanidine hydrochloride (GndHCl) and 1 mM EDTA, by placing the sample on a gyrotatory shaker for 16 to 24 hours at 4°C. The guanidine-dissolved sample was then centrifuged at 14,000 rpm for 25 minutes, to remove any undissolved proteins. The remaining undissolved protein constituted a very small pellet at the tip of the centrifuge tube (Eppendorf, Fremont, CA). Concentrations of water-soluble (WS) proteins (dissolved in Tris/HCl buffer) and WI proteins (dissolved in guanidine) were

determined with a bicinchoninic assay (BCA) protein assay (Pierce Biotechnology, Rockford, IL), using bovine serum albumin as the standard. Standard curves run either in Tris/HCl or guanidine were identical. In the BCA assay, one of the four amino acids that produces color is cystine.[56] The WI proteins are enriched in cystine compared with the WS proteins, because of the presence of a high level of disulfide-cross-linked crystallins. Thus, it is not advisable to compare amounts of protein in the two fractions (as contained in Table 1), because the level of WI protein may be artificially high compared with that of WS protein.

GndHCl-soluble proteins were fractionated by size-exclusion chromatography on an HPLC system (Shimadzu Scientific Co., Kyoto, Japan). The column was calibrated with the following protein standards (all from Sigma-Aldrich Chemical Co., St. Louis, MO): thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa). In initial experiments, the column was equilibrated with 50 mM MES buffer, 6M GndHCl, 1 mM EDTA (pH 6.2), with five times the column volume or until the OD_{280} reading attained a zero baseline. GndHCl-soluble protein was filtered with a disposable syringe filter (0.45 μm) before injection onto the column. Approximately 1 mg of dissolved WI protein per run was injected from the nucleus and approximately 0.5 mg from the cortex. Separation of proteins was performed on a 300 × 7.8-mm column (Biosep-SEC-S4000; Phenomenex, Torrance, CA), having an exclusion range of 15 to 2000 kDa. Fractionation was conducted at a flow rate of 240 μL/min.

In separate experiments, lens nuclear WI proteins were first treated with dithiothreitol (DTT) before HPLC analysis. Control and experimental proteins (1 mg) were incubated for 4 hours at room temperature in a solution of 0.27 M Tris-HCl buffer (pH 8), containing 6 M GndHCl, 1 mM EDTA, and 10 mM DTT (0.1 mL total volume). At the end of the incubation period, the proteins were analyzed by HPLC, under the conditions just described, except that the elution buffer contained 10 mM DTT.

Western Blot Analysis

HPLC fractions from pooled peaks were dialyzed overnight at 0 °C to 4°C against two changes of 3 L of 60 mM Tris/HCl buffer (pH 6.8), containing 0.1% SDS, and concentrated using ultrafiltration (10,000 MW cutoff; Amicon, Beverly, MA). Proteins were analyzed by SDS-PAGE using a 7.5% gel after reduction of −SS− in a 4× SDS gel sample buffer (0.5 M Tris/HCl [pH 6.8] containing 20% glycerol [vol/vol], 20% SDS, 1% bromophenol blue, and 50 mM DTT). Samples were boiled at 95 °C to 97°C for 5 minutes and loaded into the wells. The gel was run at a constant current of 20 mA until the bromophenol blue dye was a few millimeters from the end. Fractionated proteins were electroblotted onto polyvinylidene fluoride (PVDF) membrane overnight (Immobilon-P; Millipore Corp., Bedford, MA) using a wet transfer technique at a constant voltage (30 V) and current (90 mA). Immunodetection was conducted using a modification of the method of Padgaonkar et al.[57] Blotted samples were sequentially reacted with antibodies at the following dilutions: α-, β-, and αB-crystallins, 1:2000; γ-crystallin, 1:4000; and αA- and ζ-crystallins, 1:8000, followed by anti-rabbit IgG horseradish peroxidase. Lens crystallin antibodies produced in rabbits against calf α-, β-, and γ-crystallins and guinea pig ζ-crystallin, were gifts from J. Samuel Zigler, Jr. (Laboratory of Vision Research, National Eye Institute). Antibodies produced in rabbits against recombinant αA-and αB-crystallins were gifts from Joseph Horwitz (Jules Stein Eye Institute, UCLA School of Medicine, Los Angeles, CA). Protein bands were visualized by chemiluminescence (ECL Western Blot Detection System; GE Healthcare, Arlington Heights, IL).

Results

The effect of HBO treatment of guinea pigs on lens transparency was assessed using slit lamp biomicroscopy. After 84 O_2-treatments (7 months), lenses of experimental animals were found

to develop an increased level of nuclear light-scattering (NLS), particularly in the very center of the lens nucleus (Fig. 1B), compared with age-matched control animals (Fig. 1A). In addition, the region of backscatter appeared to be larger in the O_2-treated lens, than in the control. Treatment of animals with HBO produced a 10.5% decrease in the concentration of WS protein and a 73.8% increase in the level of WI protein in the lens nucleus (defined as the inner 25% of the lens by weight), compared with control animals (Table 1). Minimal effects (a 3.4% loss of WS protein and a 7.9% increase in WI protein) of the HBO treatment were observed for the lens equatorial cortex (the outer periphery).

SLS analysis of a control guinea pig eye in vivo clearly showed the anatomic structure of the eye: the cornea, aqueous humor, the three regions of the lens (anterior cortex, nucleus, and posterior cortex), and the vitreous humor (Fig. 2A). The high level of scatter observed for the cornea was caused by reflection of laser light from the cornea back to the detector. A low level of SLS was observed throughout the 4.5- to 5.0-mm axial diameter of the lens of the 25 month-old control animal (Fig. 2A). Lenses of guinea pigs treated 84 times with HBO showed an increased level of SLS, particularly in the nuclear region (Fig. 2B).

To evaluate changes in apparent lens protein size in vivo as a result of O_2-treatment, we conducted DLS analyses. Measurements were made every 0.1 mm across the ~5-mm optical axis of the guinea pig lens. Figure 3 shows a representative profile of average protein diameters across the lens of a control and HBO-treated animal (see Materials and Methods and the legend of Fig. 3 for a definition of average protein diameter and an explanation of why the data are expressed as arbitrary units of diameter). An increase in average protein size was obvious in the central region of the lens (the inner 2 mm) of the O_2-treated animal compared with the control. Larger apparent protein sizes were consistently noted in the central regions of lenses of experimental animals, but not always in the same region of the nucleus. Whereas in Figure 3, the highest average diameters were in the anterior and posterior regions of the experimental nucleus, certain other lenses of HBO-treated animals showed large diameters in the very center of the lens. To investigate the protein sizes in more detail, the DLS data were analyzed more thoroughly for one lens each of 13 control and 9 experimental animals (Fig. 4), again making measurements every 0.1 mm in the lens. For these determinations, apparent protein diameters (obtained as shown in Fig. 3) were averaged in three separate regions, as measured from the anterior lens capsule, including the anterior cortex (0–1.5 mm), nucleus (l.6–3.9 mm), and posterior cortex (4.0–5.0 mm). The total number of measurements made in each region in the 13 control and 9 experimental animals are shown in the legend to Figure 4. The average protein size in the nucleus of lenses of HBO-treated guinea pigs was found to be nearly twice that for the same region of lenses of age-matched control animals ($P < 0.001$; Fig. 4). No significant increases in average protein size were observed for either the anterior or posterior cortex as a result of HBO-treatment of the animals ($P > 0.1$).

It was also of interest to investigate the size distribution of proteins at certain locations in lenses of control and O_2-treated animals. A size distribution analysis was conducted in one lens each of 12 control and 9 experimental animals at 2 of the 50 in vivo lens measurement locations, in the nucleus at a point 2.0 mm from the anterior capsule and in the anterior cortex 0.5 mm from the capsule. Protein size data were averaged for control and O_2-treated lenses after dividing the sizes into small-diameter (>50 arbitrary units) and large-diameter (<50 arbitrary units) proteins (Table 2). In the nucleus, at the 2.0-mm location, lenses of control animals showed 90% of the total protein intensity clustered into the group of small-diameter polypeptides (mean diameter, 18 arbitrary units). A second, less prominent control group, making up 10% of the total intensity, had a size approximately 16 times that of the major group (mean diameter, 279 arbitrary units). Results for the O_2-treated lenses at the 2.0-mm location (Table 2) showed two prominent groups of proteins. The O_2-treated small-diameter proteins, making up 68% of the total intensity, had a size (mean diameter, 29 arbitrary units) 1.6 times that of the major control

group ($P > 0.001$), and the O_2-treated large-diameter proteins, accounting for 32% of the total intensity, had a size (mean diameter, 528 arbitrary units) 30 times the control small proteins and nearly twice the control large proteins ($P = 0.03$). Thus, at this one location in the lens nucleus, the oxygen treatment produced a threefold increase in intensity and a twofold increase in size of the large, aggregated proteins. In the anterior cortex at the 0.5-mm location (Table 2), there were no significant differences in the intensities and apparent sizes of the two groups of proteins, control versus O_2-treated.

Aggregation of proteins in the lenses of O_2-treated guinea pigs was also investigated by HPLC analysis of lens WI proteins. The proteins were dissolved in 6 M GndHCl, taking care not to produce any artifactual protein disulfide (see Materials and Methods), but initially without the use of disulfide-reducing agents. In addition, proteins were eluted initially under nonreducing conditions. The profile for the nuclear region of the control lens (Fig. 5A) showed two well-defined peaks, a minor higher molecular weight Pk1 (37.0 minutes of elution) and a more prominent lower Pk2 (40.5 minutes of elution). In contrast, the experimental nuclear profile showed a 50% decrease in the amount of the lower molecular weight fraction Pk2, plus a shift of this peak to a 10% higher molecular weight (from 40.5 minutes of elution to 39.5 minutes), as well as a substantial increase and shift to a higher molecular weight for Pk1. Nearly one half of the experimental nuclear WI protein fraction that had been dissolved in guanidine consisted of 150 to 1000 kDa proteins (the higher shoulder of Pk1, 25–37 minutes of elution) that were not present in the control (Fig. 5A). Only a minimal difference was observed between the chromatographic pro-files for control and experimental lens cortical WI proteins dissolved in guanidine (Fig. 5B).

When the experimental nuclear protein sample of Figure 5A was reduced with 10 mM DTT before application to the HPLC column, nearly all the proteins were found to elute at significantly lower MWs (Fig. 6, solid line), compared with results obtained without DTT-reduction (Fig. 5A, solid line). Pk1 shifted from a broad elution-time range of 25 to 37 minutes (Fig. 5A, solid line) to 44 minutes (Fig. 6, solid line); and, similarly, Pk2 shifted from 40 minutes to 46.5 minutes. There was also a shift in the elution times for the DTT-treated control nuclear WI proteins: Pk1, 37 to 43 minutes; Pk2, 40.5 to 47 minutes (Figs. 5A, 6, dashed lines). It is not clear whether this DTT-induced shift of the control WI proteins to slower elution times was caused by actual reduction of disulfide or by some other effect of DTT on the protein elution rate. We believe the latter possibility to be true, since treatment of αB-crystallin by itself with DTT causes a similar shift (data not shown), and αB-crystallin is known to contain no −SH groups.

Studies were conducted to identify the crystallins present in the high-molecular-weight (HMW) fraction, as well as Pk1 and Pk2, of the HPLC elution profile of Figure 5A (lens nuclear WI proteins dissolved in GndHCl, without DTT). The fractions were pooled (the experimental Pk1 included the 150 to 1000 kDa aggregated material, but not the HMW fraction), dialyzed, concentrated, and analyzed by SDS-PAGE and Western blot analysis after reduction of the sample with DTT. Western blot results for the experimental fractions (solid line of Fig. 5A) are shown in Figure 7. Regarding the HMW and Pk1 fractions, all the crystallins, except αB-crystallin, were found to be present. These included αA-, β-, γ-, and ζ-crystallins. For the lower molecular weight fraction Pk2 of Figure 5A (solid line), all the crystallins, except ζ-crystallin, were present. These included αA-, αB-, γ- and ζ-crystallins. The experimental HMW fraction also contained a 40 kDa γ-crystallin protein (Fig. 7, arrowhead) that was resistant to reduction by DTT and not present in either the Pk1 or Pk2 fractions. Similarly, Pk1 contained a 42 kDa β-crystallin polypeptide (Fig. 7, arrow) resistant to DTT, and not present in the HMW or Pk2 fractions. Western blot analysis of the control Pk1 and Pk2 of Figure 5A (dashed line; WI protein dissolved in guanidine from the nucleus of lenses of age-matched control animals) were identical with the experimental results and are not shown. There was an insufficient amount

of control HMW fraction to conduct an analysis. Western blot analysis of both the control and experimental WI protein samples before HPLC injection showed a significant amount of 46 kDa α-crystallin that was resistant to DTT reduction (data not shown). This protein band was not seen in any of the other fractions, and is presumed to have been trapped in the pre-HPLC filter.

Discussion

Treatment of guinea pigs with HBO accelerated the formation of WI proteins in the lens nucleus, but not in the cortex (Table 1). The results raise the possibility that molecular oxygen may also be linked with the loss of WS proteins that takes place in the aging human lens nucleus. Such a loss is a major indicator of aging in the center of the lens, and is a hallmark in the development of human nuclear cataract.[58] Additional evidence of possible O_2-induced changes occurring in the center of the aging human lens is the finding that oxidation of cysteine and methionine residues are the major modifications found in WI proteins of those lenses.[52] Oxygen is present in the lens nucleus at a level of approximately 1%,[17] a concentration that may increase with age as a result of liquefaction of the vitreous humor, possibly permitting more of the oxidant to enter the lens by the retinal circulation.[23] In addition to O_2, all other components required for nonenzymatic metal-catalyzed oxidation are present in human lens fiber cells, including ascorbate, H_2O_2, and catalytic metals, such as copper and iron. [59] Compared with the cortex, the lens nucleus does not possess a strong antioxidant defense; for example, it has no activity of the hexose monophosphate shunt which would be needed to produce the reductant NADPH,[13] and normal diffusion of the antioxidant GSH from the cortex into the nucleus may be blocked in older lenses due to the formation of a yet-unidentified barrier.[60]

Animals in this study received a total of 200 hours of elevated oxygen exposure (over a 7-month period), producing an increase in lens nuclear light-scattering as observable by slit lamp (Fig. 1). This effect on lens transparency represented an early stage in the formation of nuclear cataract and was not a dense opacity. Humans who were treated therapeutically with HBO for a total of 400 hours, twice as long as the time of the present study, showed development of nuclear cataracts.[24] Previous studies of human nuclear cataracts have indicated that a relatively small number of scattering bodies within the lens nucleus, without a significant amount of cellular damage, may be sufficient to cause visual impairment.[38] Benedek et al.[46] have discussed how small increases in the diameter of aggregates in the lens can produce very large effects on the amount of light that is scattered. One factor, not considered in the present study, was the amount of light scattered forward toward the retina of the guinea pig eye; this light is considered to be more of a cause of reduced vision compared with light that is backscattered.[61]

DLS analysis conducted at 50 different locations across the optical axis of the guinea pig lens showed an O_2-induced increase in apparent protein size in the nuclear region (Fig. 3). Although the increase in particle size was confined to the central 2-mm diameter of each lens analyzed, the effects were often found to be inconsistent throughout the region. For example, in Figure 3, particle sizes in the very center of the experimental lens are shown to be two to three times lower than those in the anterior and posterior region of the nucleus. For the other eight experimental lenses analyzed, larger protein sizes were frequently seen at certain locations in the nucleus, including the anterior, central, or posterior areas, but sometimes not in other parts of the nucleus. The reason for this is not clear, but the data suggest that early O_2-induced aggregation of proteins may not progress homogeneously throughout the nucleus, thus resulting in the formation of larger aggregates in certain regions of the lens nucleus, and smaller aggregates in other regions. It is also possible that if O_2-induced aggregates had become too large, multiple light-scattering (a single photon scattering many times in the lens) would have

occurred, giving rise to absorption of light, and causing diffusion broadening, which could produce apparently lower particle sizes. This might explain the relatively lower protein sizes seen in the very center of the experimental lens in Figure 3. Considering the equatorial diameter of the lens (rather than the axial diameter), previous in vitro laser scanning of lenses of HBO-treated guinea pigs showed increased scatter along the central 2 mm of equatorial length.[62] Because the length of the focused laser light beam used in the present study was 20 μm, and measurements were made only along the optical axis, only 1% of the total 2-mm region of scatter along the equatorial length of approximately 20 lens fiber cells would have been analyzed at each of the measurement points.

DLS analysis showed an average doubling in apparent protein size in the central 2-mm region of lenses of O_2-treated guinea pigs (24 measurements per each lens nucleus along the optical axis), compared with age-matched control animals (Fig. 4). It is not clear whether this degree of aggregation could have produced the level of scatter observed by slit lamp examination. The slit lamp–detected scatter may have been produced by a relatively small number of much larger aggregates. Size-distribution analysis conducted at one of the 24 measurement points in the lens nucleus (2.0 mm in from the anterior capsule) in nine experimental animals showed that the group of large-diameter proteins (>50 arbitrary units) increased threefold in intensity and doubled in size, compared with the age-matched control animals (Table 2). The average diameter (528 arbitrary units) of the O_2-induced aggregates at this one location was 30 times greater than that of the control small-diameter proteins (18 arbitrary units). If a 5-nm average size is assumed for the control small-diameter proteins (approximately 40% of this fraction should have consisted of 10- to 12-nm α-crystallin), an approximate diameter of 150 nm can be estimated for the O_2-induced aggregates (Table 2). Aggregates of this size would have most certainly produced significant scattered light in the guinea pig lens. Other investigators, using techniques of electron microscopy or light-scattering, have observed aggregates with 100- to 500-nm diameters in human nuclear cataracts.[29,37,38,46,63,64] Electron microscopic studies of aggregates in experimental animal cataractous lenses showed sizes of 100 nm in the HBO-treated guinea pig,[37,38] and even up to 1000 nm in the X-irradiated rabbit.[65]

Table 2 shows that small-diameter proteins present in the O_2-treated nucleus (average diameter, 29 arbitrary units) were 1.6 times the size of the same group of proteins in the control nucleus (average diameter, 18 arbitrary units). It is conceivable that some of this experimental fraction may have represented chaperone-like activity of α-crystallin,[66] involving the binding of oxidatively damaged crystallins to the α-crystallin complex by hydrophobic interaction.[67] α-Crystallin has been shown to decrease the amount of light-scattering and thiol oxidation of other crystallins induced by various conditions of oxidative stress in vitro,[68] and rabbit lenses treated with HBO in vitro have exhibited an apparent chaperone-like function for α-crystallin in the lens nucleus.[69] In the aging bovine lens, α-crystallin present in the HMW fraction has been shown to bind β- and γ-crystallins in the central region of the supramolecular complex.[70]

Nearly one half of the experimental nuclear WI protein dissolved in guanidine was found to consist of 150- to 1000-kDa proteins that were not present in the control (Fig. 5A). Almost all this aggregated material appeared to be held together by disulfide bonds (based on the DTT reduction experiments of Figs. 6, 7). For aggregates of this size to have existed in guanidine, 7 to 50 individual 20-kDa MW crystallin subunits would have had to be cross-linked by disulfide bonds. It is not known what the sizes of these aggregates might have been in vivo (before denaturation with guanidine), but the material may have corresponded to the very large aggregates that were detected in the experimental nucleus in vivo with the use of DLS (Table 2). As mentioned earlier, those aggregates may have been up to 150 nm in diameter. Bettelheim et al.[64] have calculated that a 300-nm diameter aggregate would have a molecular mass of 500×10^6 Da. Aggregates up to 300×10^6 Da have been found to exist in the water-soluble fraction

of old, normal human lenses.[40] As mentioned previously, the diameters of aggregates found in cataracts have ranged from 100 to 1000 nm.

It appeared that when nuclear WI proteins present in control and O_2-treated lenses were dissolved in guanidine, they consisted of both disulfide-cross-linked aggregates (Fig. 5A, Pk1) and monomers (Fig. 5A, Pk2). The monomers apparently had become WI for some reason other than direct oxidation of −SH groups (for example, they could have been initially bound hydrophobically to a cross-linked aggregate and then released as monomers during treatment with guanidine). Supporting evidence for this is that αB-crystallin, which does not contain any −SH groups, was found to be present only in the Pk2 fraction and not at all in the disulfide cross-linked material of Pk1. In addition, ζ-crystallin, which contains five −SH groups per subunit, was present only in Pk1 and not at all in Pk2 (Fig. 7). In a previous study, disulfide cross-linking was the major modification present in the WI protein fraction of old, normal human lenses[52] and, in x-ray cataracts, 50% of −SH groups present in the soluble HMW aggregate fraction were oxidized.[71] Although in the present study, treatment with DTT reduced all the very HWM material (possessing molecular masses in guanidine of >150 kDa; the shoulder of Pk1, Fig. 5A), 50% of the reduced material was found to remain in Pk1, presumably mostly as smaller disulfide-cross-linked aggregates (Fig. 6). This may have been caused by DTT not being able to reach all the disulfide cross-links during the 4-hour incubation at room temperature. For Western blot studies (Fig. 7), in which samples were reduced with DTT at 95 °C to 97°C, nearly all the aggregated material was reduced to monomers.

The results of this study support the long-standing belief that disulfide bonding may be the main cause of HMW aggregate formation in human nuclear cataracts.[7,8] Disulfide has also been linked with HMW aggregate formation in certain experimental cataracts such as those induced by x-ray[71] and diabetes.[72] The cysteine residue is known to be highly sensitive to oxidation by almost all kinds of reactive oxygen species.[73] The fragile nature of the −SH to −SS− relationship in the human lens is demonstrated by an Arg-14 to Cys mutation in the lenses of human infants, causing hereditary cataract as a result of disulfide cross-linked aggregate formation.[74] Why the mammalian lens would require such a high −SH content (nearly 50 mM), when cysteines are so susceptible to oxidation and cross-linking, is not known. It has been proposed that cysteine may be needed to interact with aromatic-side chains in the lens, quenching excited states as crystallins absorb ultraviolet light, protecting the retina from damage.[75]

In addition to disulfide-cross-linking, Western blot analysis indicated a relatively lesser amount of nondisulfide covalent cross-linking in the control and experimental WI protein fractions (Fig. 7). A 46 kDa αA-crystallin polypeptide was detected at a substantial level in the guanidine-dissolved protein sample before HPLC-injection (data not shown), but was not found at all in any of the HPLC fractions. This polypeptide may have represented a nondisulfide cross-linked αA-crystallin dimer present in aggregates that, even in 6 M guanidine, were too large to enter the HPLC column. The nondisulfide cross-linked 40-kDa γ-crystallin present in the guanidine-dissolved HMW fraction (Fig. 7) apparently existed in very large disulfide cross-linked aggregates in the experimental WI protein. A nondisulfide cross-linked 42-kDa β-crystallin was present only in Pk1 (Fig. 7). In a previous study, a 43-kDa polypeptide with immunoreactivity to β-crystallin antibody was isolated from human nuclear cataracts and proposed to be a possible nucleation site for the generation of disulfide cross-linked HMW membrane-bound aggregates.[76] The nature of the nondisulfide covalent cross-links mentioned earlier is not known. It is possible that some of the cross-links are related to transglutaminase activity, which has been reported to be present in the lenses of both the guinea pig and human.[77] Transglutaminase-mediated cross-linking of both α- and β-crystallins has been shown to take place under certain conditions, including elevated oxidative stress.[78,79]

All the guinea pig lens crystallins, including α-, β-, γ-, and ζ-, were found to be present in the WI protein fraction of the experimental lens nucleus (Fig. 7). Both WI proteins and water-soluble protein aggregates in human lenses have been found to contain α-, β-, and γ-crystallins.[40,52,80] As discussed earlier, αB-crystallin with no −SH content may have been present in the WI fraction indirectly as a result of hydrophobic bonding to disulfide cross-linked αA-crystallin. The β- and γ-crystallins possess substantial −SH content and are known to be prone to insolubilization as a result of exposure to oxidative stress.[7,69,81,82] ζ-Crystallin, an NADPH-binding protein making up 10% of the total soluble protein in the guinea pig lens[83] and containing five −SH groups per subunit, was found in the present study to be particularly vulnerable to O_2-induced disulfide-cross-linking. ζ-Crystallin was the only crystallin in the WI fraction of the experimental nucleus to be present entirely in the disulfide cross-linked aggregate fraction of Pk1, with no protein existing in the monomer fraction Pk2 (Fig. 7). The same result was found for the WI fraction of the control nucleus, implying that the only mechanism by which ζ-crystallin becomes insoluble in the guinea pig lens is through disulfide cross-linking.

The crystallin composition of WI protein fractions in control and O_2-treated guinea pig lenses did not differ significantly after 84 treatments of the animals with HBO. The dramatic difference between the two fractions was the substantially greater amount and size of disulfide cross-linked aggregates contained in the experimental WI protein, compared with the control. This suggests that an elevated level of oxygen in the lens may act to accelerate normal oxidative changes in crystallins in the center of the lens as lens nuclear proteins age in a nonregenerating environment.

In summary, we have shown by in vivo and in vitro analysis that HBO-treatment of guinea pigs induces HMW aggregate formation in the center of the lens, leading to increased nuclear light-scattering. A similar process of O_2-induced disulfide cross-linking of crystallins may be involved in the formation of human nuclear cataract.

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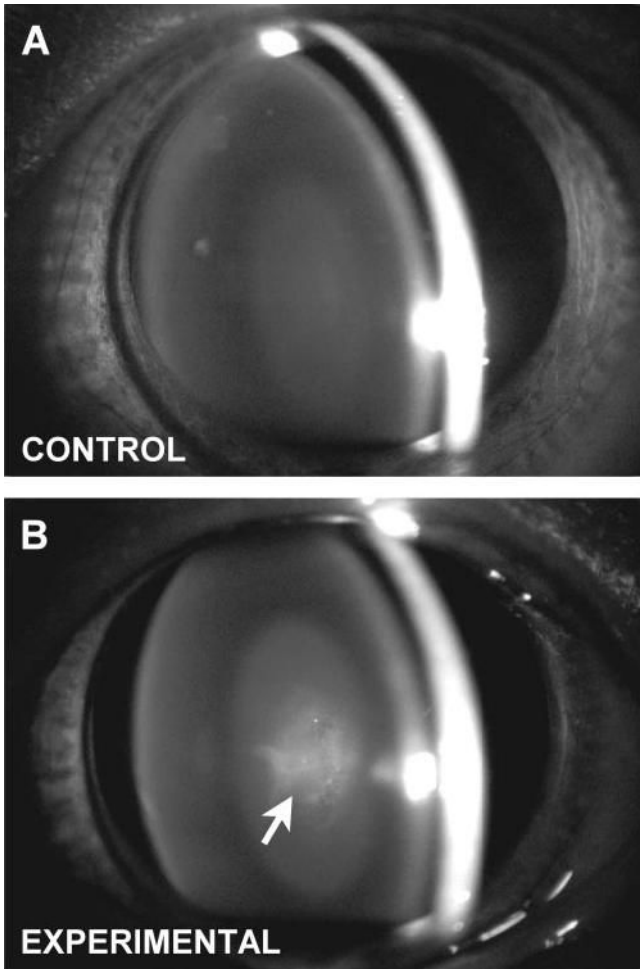

Figure 1.
Typical slit lamp biomicroscopy photographs of guinea pig eyes. (**A**) A 25-month-old control animal. (**B**) A 25-month-old animal after 84 treatments with HBO over a 7-month period. Note the increased lens nuclear light-scattering in the O_2-treated lens, compared with the control, particularly in the very center of the nucleus (*arrow*). In addition, note the larger region of backscatter in the O_2-treated lens, compared with the control.

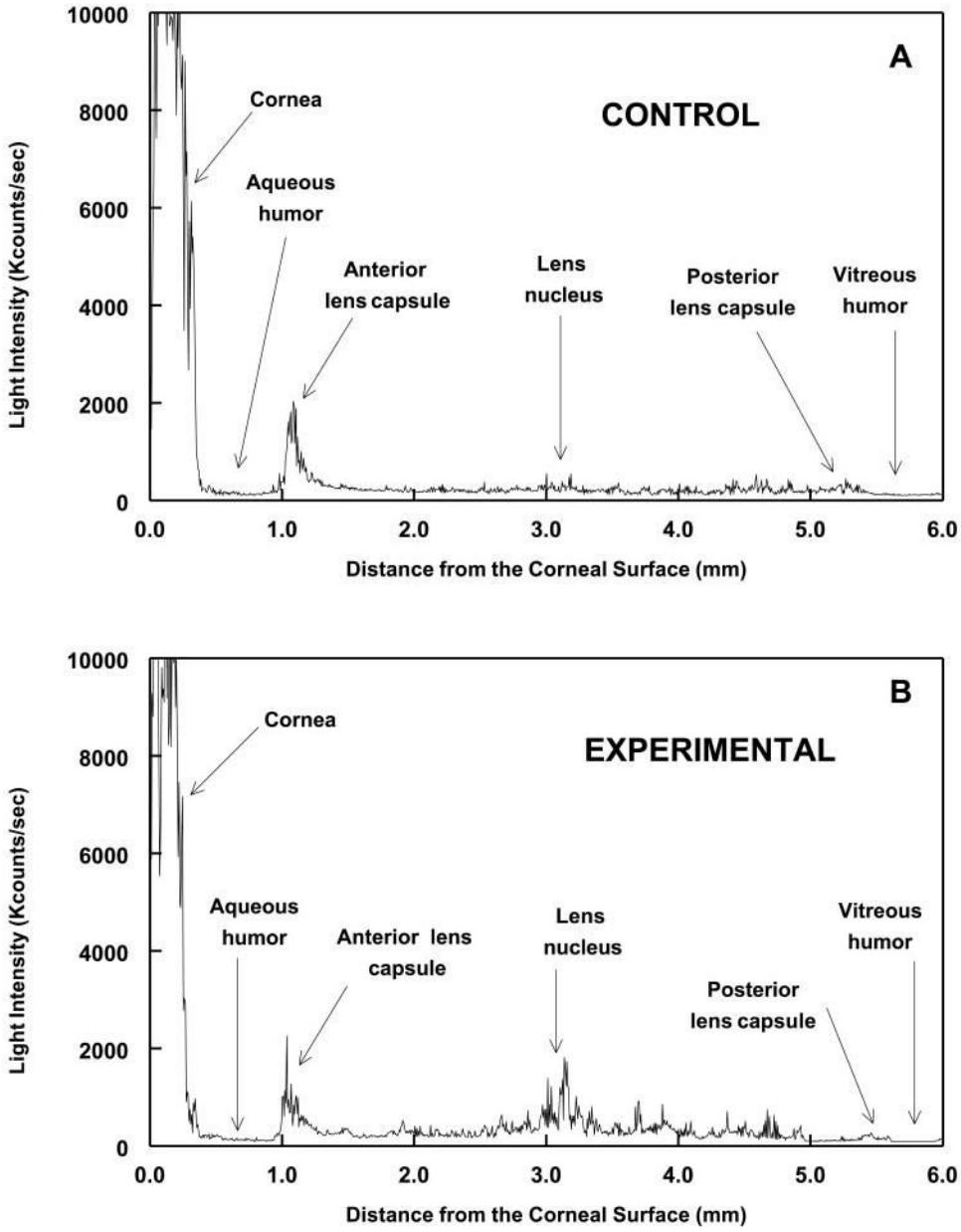

Figure 2.

Typical SLS analyses in vivo of eyes of control and HBO-treated 25 month-old guinea pigs. Each analysis was conducted along the optical axis of the lens, measuring 4.5 to 5.0 mm in diameter. *K* counts/s: 1000 photon counts per second of light intensity. (**A**) Control, untreated animal; (**B**) animal treated 84 times with HBO. Note the increase in SLS intensity in the experimental lens, particularly in the central region, compared with the age-matched control.

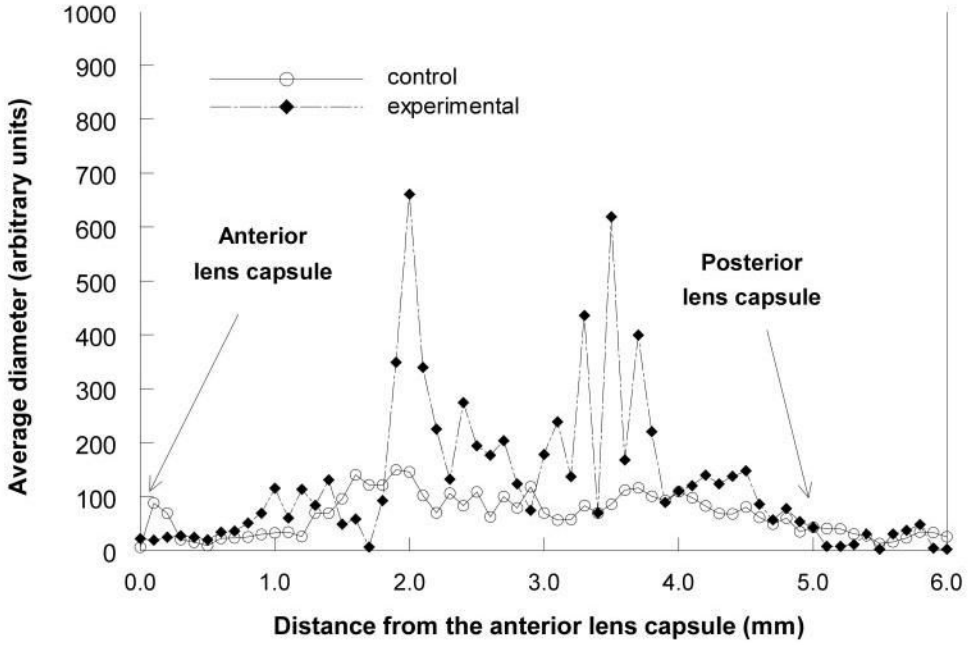

Figure 3.

Representative DLS analysis profiles in vivo across lenses of control and HBO-treated guinea pigs (not the same animals reported in Fig. 2). Measurements of average protein diameter were made every 0.1 mm across the approximate 5-mm optical axis of each lens. The instrument made 10 to 15 measurements of protein diameter at each point and provided an average value. The DLS data are not expressed as absolute sizes of lens proteins but as arbitrary units of diameter (see Materials and Methods for an explanation). (○) 25-month-old control animal; (♦) 25-month-old animal after 84 treatments with HBO.

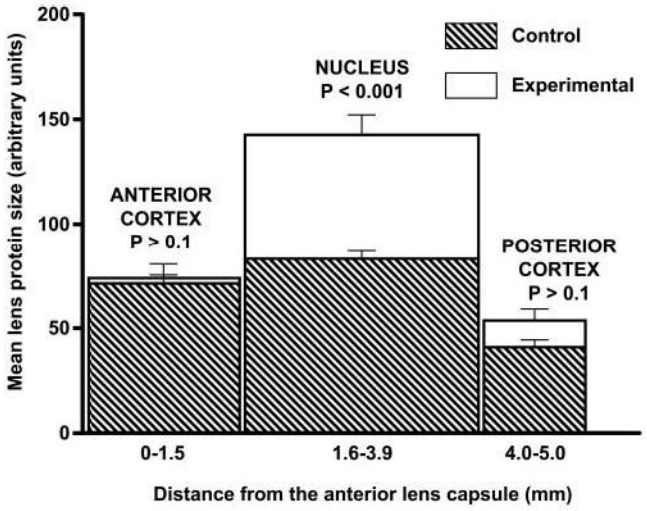

Figure 4.

Analysis of in vivo dynamic light-scattering data for measurements taken across the lenses of 25-month-old control and HBO-treated (84 treatments) guinea pigs. Measurements of average protein diameter were made every 0.1 mm across the approximate 5-mm optical axis of each lens (see the legend of Fig. 3 for an explanation of how this was done). These data were then averaged for three regions of one lens each for 13 control and 9 experimental animals. The total number of measurements were: anterior cortex, control: 224, experimental: 144; nucleus, control: 312, experimental: 215; and posterior cortex, control: 143, experimental: 97. Results are expressed as the mean ± SEM.

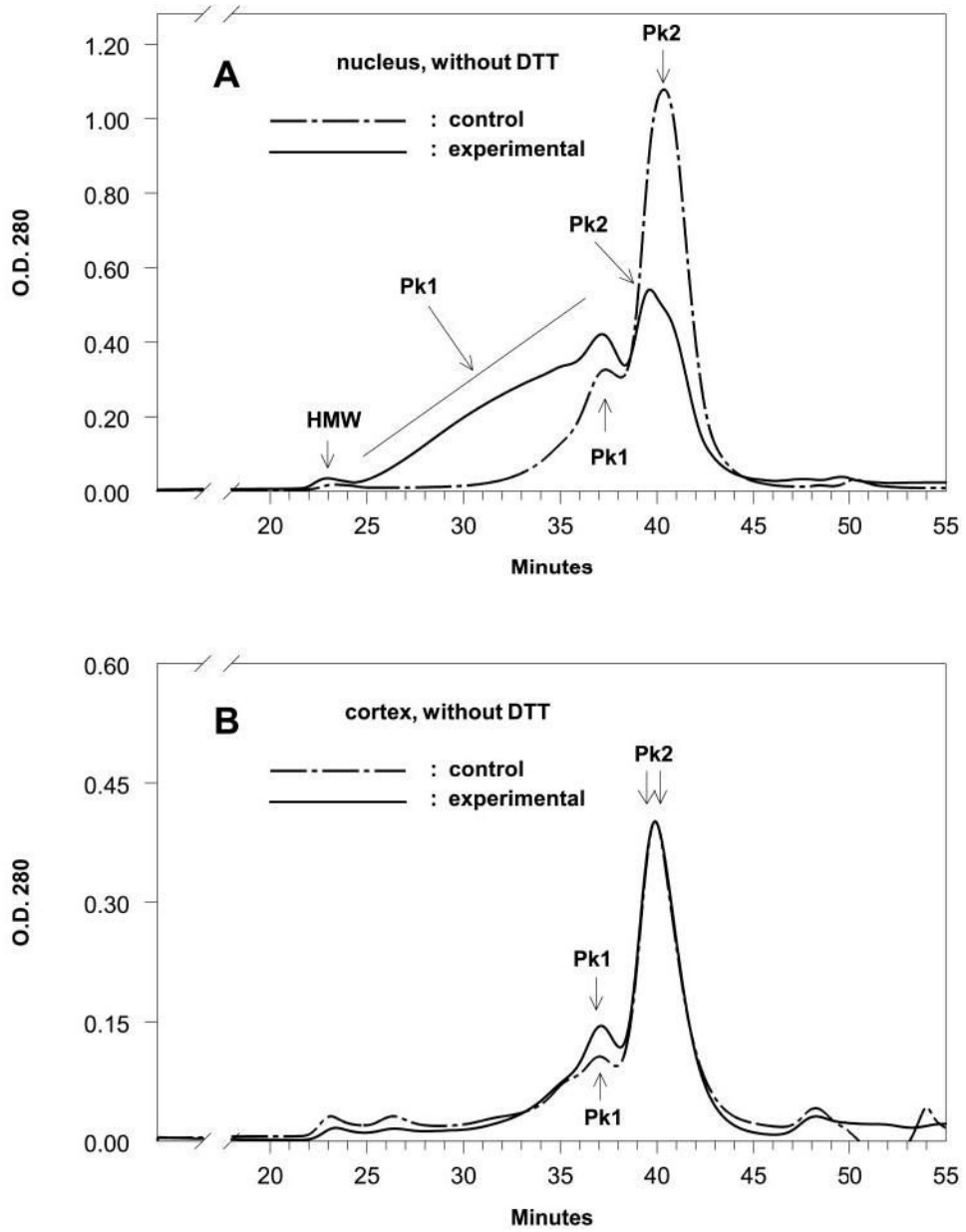

Figure 5.

HPLC elution profiles of guinea pig lens WI proteins dissolved in 6 M GndHCl under nondisulfide-reducing conditions. Proteins were separated by gel filtration under non-reducing conditions at a flow rate of 240 μL/min. The profiles shown are representative of analyses of lenses from three 25-month old control animals or experimental animals treated 84 times with HBO. (**A**) Lens nucleus (1 mg protein applied for both the control and experimental). (**B**) Lens cortex (0.5 mg protein applied for both the control and experimental).

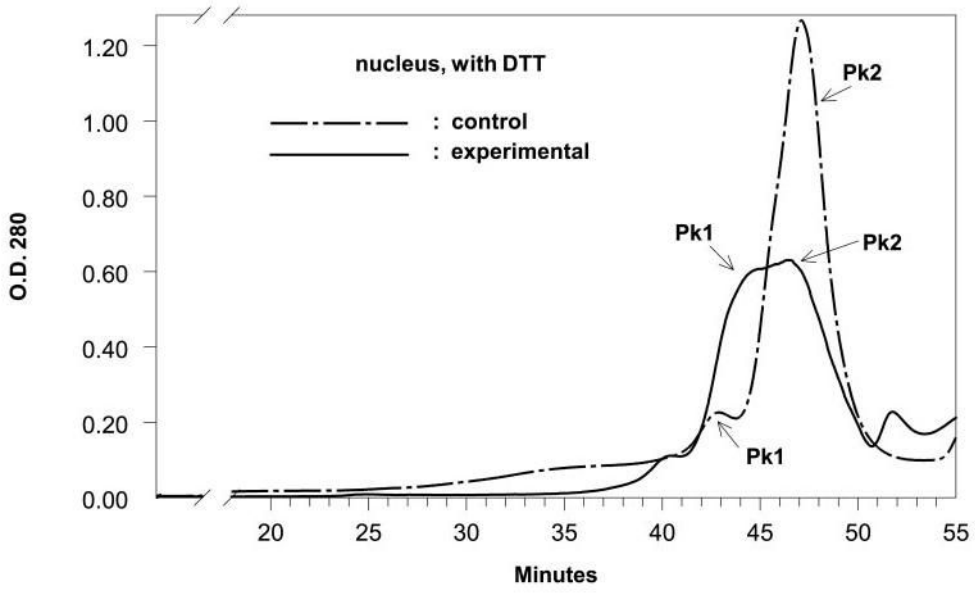

Figure 6.

HPLC elution profiles for the control and experimental nuclear samples of Figure 5A (WI nuclear proteins dissolved in 6 M GndHCl) after reduction of the sample with DTT. Proteins (1 mg) were separated by gel filtration under reducing conditions at a flow rate of 240 μL/min. The profiles shown are representative of analyses of lenses from three animals 25-month-old control animals or experimental animals treated 84 times with HBO.

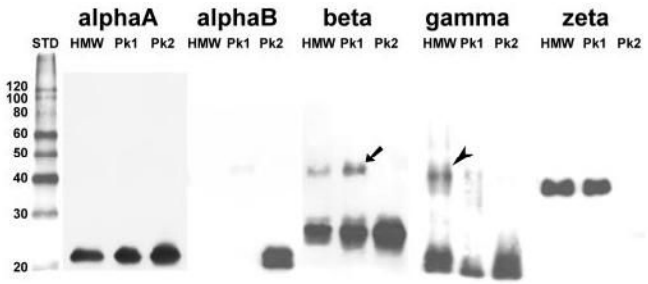

Figure 7.

Western blot analysis of the HMW, Pk1 and Pk2 fractions contained in the experimental HPLC elution profile of Figure 5A (WI nuclear proteins dissolved in guanidine, without DTT, from the lenses of guinea pigs treated 84 times with HBO). Fractions were pooled (HMW: 22–24 minutes elution; Pk1: 25–37 minutes elution; and Pk2: 39–42 minutes elution), dialyzed, concentrated, and analyzed after reduction of disulfide with DTT. Immunostaining was conducted using antibodies to αA-, αB-, β- γ-,, and ζ-crystallins. *Arrowhead* and *arrow*: indicate polypeptides resistant to DTT reduction. Note the absence of αB-crystallin in the HMW and Pk1 fractions and the absence of ζ-crystallin in the Pk2 fraction.

Table 1

Effect of HBO Treatment In Vivo on the Concentration of WS and WI Proteins in the Guinea Pig Lens

Conditions	Lens Cortex		Lens Nucleus	
	WS	WI	WS	WI
Control	358.0	17.8	308.0	126.7
HBO-treated	346.0	19.2	275.7	220.2
Change from control	3.4%↓	7.9%↑	10.5%↓	73.8%↑

Data are expressed as milligrams of protein per gram of wet weight. Guinea pigs were treated 84 times with HBO over a 7-month period. Control and experimental animals were 25 months old at the time of analysis. Lens cortical and nuclear tissues were pooled from the two lenses each of two control and two experimental animals. The nucleus was defined as the inner 25% of the total lens weight. The cortex was the equatorial region of the lens, amounting to 55% of the total lens weight. As explained in the text, it is not advisable to compare amounts of WS and WI proteins; however, amounts of WI protein in the nucleus and cortex can be compared.

Table 2

Protein Size Distribution Analysis Conducted In Vivo

	Intensity (%)		Diameter (Arbitrary Units)	
	Control	Oxygen	Control	Oxygen
2.0 mm (nucleus)				
Small diameter	90 ± 8	68 ± 15	18 ± 3	29 ± 6
	$P < 0.001$		$P < 0.001$	
Large diameter	10 ± 8	32 ± 15	279 ± 246	528 ± 235
	$P < 0.001$		$P = 0.03$	
0.5 mm (cortex)				
Small diameter	93 ± 7	92 ± 8	10 ± 3	11 ± 2
	$P > 0.1$		$P > 0.1$	
Large diameter	7 ± 8	8 ± 6	196 ± 307	84 ± 16
	$P > 0.1$		$P > 0.1$	

Data were collected at two points in lenses of control and HBO-treated guinea pigs, 2.0 and 0.5 mm from the anterior capsule. In vivo DLS analyses were conducted after 82 treatments of guinea pigs with HBO. Control and O_2-treated animals were 25 months old at the time of the analyses. Small-and large-diameter proteins were classified as being <50 or >50 arbitrary units, respectively. The text and the legend of Figure 3 explain why the data are expressed as arbitrary units of protein diameter. Results are expressed as the mean ± SD for 12 control and 9 O_2-treated animals.